ULTRASTRUCTURAL AND PHYSIOLOGICAL CHANGES IN THE CRYOPRESERVED SPERMATOZOA OF *TOR PUTITORA* (HAMILTON)

Ravindragouda Patil[1*], W. S. Lakra[2], Shrinivas Jahageerdar[2], Gopal Krishna[2] and A. K. Pal[2]
[1]Fisheries Research and Information Center, KVAFSU, Hessaraghatta, Bengaluru- 560089, Karnataka State, India.
[2]ICAR-Central Institute of Fisheries Education, Panch Marg, Versova, Mumbai, Maharastra, India.
*Corresponding Author, E-mail: ravi.patil30@gmail.com
Telephone : +91-7204313896
Received : 28.12.2015; Accepted : 20.06.2016

ABSTRACT

Cryopreservation of spermatozoa of golden mahseer, *Tor putitora*, was carried and the ensuing ultrastructural and physiological changes in the spermatozoa were studied by electron microscopy and Nitro Blue Tetrazolium (NBT) assay. The spermatozoa density and optimum sperm: egg ratio was estimated to be 3.96 ± 0.12 X 10^7 spermatozoa/ ml and 3.96 ± 0.12 x 10^4 No. per egg, respectively. Cryopreservation of the spermatozoa was carried using modified BWW as an extender and a combination of 9% DMSO and 11% glycerol as cryoprotectants. After 30 days of cryo-storage, fertility evaluation of cryopreserved spermatozoa was carried and a hatching percentage of 45.97 ± 1.72 was recorded. Ultrastructure of fresh spermatozoa revealed a spherical head (diameter 1.97 ± 0.016), smaller mid-piece (length 0.31 ± 0.003), an elongated tail (length 31.10 ± 0.133) and a typical 9+2 tail fiber arrangement. Ultrastructural damages to spermatozoa following cryopreservation included, loose chromatin, disruption in cytoplasmic membrane of the head, mid-piece and tail. NBT assay recorded a lower absorbance value of 0.15 ± 0.005 for cryopreserved spermatozoa as compared to that of fresh spermatozoa (0.33 ± 0.012) indicating damage to the mitochondrial enzyme complex. It was also suggested that the Sperm Mitochondrial Activity Index assessment (SMAI) may be employed as an additional biochemical test to assess the accuracy of cryopreservation and also as an indicator of fertility status of fish milt.

Keywords: ***Tor putitora*, Spermatozoa, Cryopreservation, Ultrastructure, NBT**

INTRODUCTION

Tor putitora, popularly known as Golden Mahseer belongs to family Cyprinidae. Golden Mahseer along with other *Tor* species was a major fishery of the rivers and streams of India till a few years back. Due to over fishing, pollution, loss of habitat and other anthropogenic factors today Mahseer are enlisted as a group of endangered fishes (Mahanta *et. al.,* 1988; Ogale, 1994; Basavaraja and Keshavanath, 2000). The reproductive

traits like low fecundity, short spawning season and high mortality among fertilized eggs and larvae have aggravated problem further. Considering the economic and ecological importance of *T. putitora* there is an urgent need to conserve and propagate this species further. Cryopreservation of spermatozoa is one of the important *ex situ* methods of conservation of species and its genetic diversity (Chao and Liao, 2001; NBFGR, 2000; Patil and Lakra, 2005; Rao, 1989). Cryopreservation causes damages to the cell and cell structures, and alters their physiology and functions, collectively termed as cryo-injuries. To develop optimum cryopreservation protocol which minimizes the cryoinjuries it is essential to study the effect of cryopreservation-thawing on the structural and functional integrity of the fish spermatozoa and its structures, especially the mitochondria (Figueroa *et al*., 2015).

Ultrastructure study of the of fish spermatozoa is essential to understand the sperm quality and relate it to the other quality parameters like sperm motility and velocity which are common indicators of sperm fertility (Jamieson and Leung, 1991). Ultrastructure of fish spermatozoa also helps to understand the types of damages the spermatozoa suffers during the cryopreservation (Billard *et. al*., 2000; Butts *et al*., 2010; Hatef *et al*., 2010). Normal physiology of the sperm helps it to be motile to enable it to reach and fertilize the ovum, thereby transferring the paternal genome to the ovum. The motility of the sperm cell is achieved by the sliding motion of the microtubules of axoneme of the flagellum/tail which involves expenditure of Adenosine Triphosphate (ATP). ATP hydrolysis catalyzed by dynein–ATPase of the axoneme causes motility of the flagellum/tail. Mitochondria present in the mid-piece, generate energy in the form of ATP through complex biochemical processes with the help of mitochondrial enzyme complex including ATP-synthase (Cosson, 2012).

The present study was aimed to evaluate the fertility, morphology, ultrastructural and physiological changes in cryopreserved-thawed spermatozoa of *T. putitora*.

MATERIAL AND METHODS

Present investigation was undertaken in the mahseer conservation farm and hatchery complex of *T. putitora* 'Tata Power Company' Lonavala, Maharashtra, India. The region receives major rainfall during the South-West monsoon from mid-June to September and the ambient temperature of the water ranges from 18-24^{0}C. The mature males and females of the species are found to be in oozing condition during October to December months.

Collection of milt and ova

Mature males and females of *T. putitora* were caught, by cast nets from the broodstock ponds. Fishes in oozing condition and good health were selected. The genital aperture, anal fin and the fish

belly were rinsed with sterile modified Krebs-Ringer's solution, hereafter referred to as BWW medium (Biggers *et al*., 1971; Ravinder *et al*., 1997) and care was taken to collect milt and egg samples without contaminating with fecal matter, blood or scales. The milt was collected from individual males by hand stripping in separate, clean, sterile, dry and graduated 15 ml centrifuge tubes. The egg samples were collected separately in 250 ml beakers. The collected milt and egg samples were stored on ice in a styrofoam box and transported to the laboratory at the hatchery complex.

Estimation of sperm density and motility percentage

The density of spermatozoa in fresh milt collected from the twelve male fishes was estimated with the help of improved Neubauer Haemocytometer (Ax *et al*., 2000) and by using the formula, Spermatozoa /ml = Cells in five group squares (in 16 x 5 = 80 squares) / (80 x 1/400 x 1/10 x 1/1000). Motility percentage of spermatozoa was estimated as per the procedure suggested by Billard *et al*. (2000). Modified BWW extender was used to dilute the milt. The percentage of motile spermatozoa was estimated by formula, % Motile Sperms = [(Total no. of spermatozoa-Total no. of non-motile spermatozoa) / (Total number of spermatozoa)] x 100.

Optimization of sperm: egg ratio

Eggs were collected from mature females by hand stripping. Milt samples with more than 70% motility were pooled and diluted with sterile, modified BWW extender to get a dilution of 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} as per Suquet *et al*. (1995). Approximately, 0.5 ml eggs not hardened by water were taken in a sterile 2 ml plastic syringe without the needle and distributed in sterile petri plates. Triplicates were maintained for each dilution and for the neat milt. About 0.5 ml of neat and diluted milt were dispensed on these eggs and mixed gently with a blunt glass rod with flattened and smooth edge and 4 ml of fresh pond water was added. After about 30-60 seconds, some more water was added to these petri plates to remove excess milt. The actual number of the eggs in each petri plate was counted and the petri plates were placed in wooden trays of dimension 0.56m x 0.56m x 0.10m, with nylon mesh (mesh size of 1mm). The trays were floated over water in small cement cisterns of 2.5m x 1.2m x 0.75m dimension. Fresh water from dam was pumped into an overhead tank and jets of water were sprayed from both sides of the cement cistern over the hatching trays to provide aeration as well as to maintain the flow-through system of water supply. Dead eggs were removed at 6 hourly intervals and after 24 hours of incubation, the numbers of live embryos were noted down. The eggs were incubated for 72+6 hours and the hatchlings were counted and hatching percentage was calculated.

Cryopreservation of milt and its evaluation

The collected milt samples were

pooled and the sperm density was estimated (Ax *et. al.*, 2000). French medium straws (0.5 ml) were used to cryopreserve the milt. Cryopreservation of the milt was carried as per Patil and Lakra (2007). After freezing, the straws were stored in LN2 cryocans (IBP Ltd.) for a minimum period of 30 days and after that its fertility was evaluated as per Suquet *et al*. (1995). The cryopreserved milt was thawed by immersing the straws fully into the water maintained at 37 ± 1^0 C for 10 seconds. The percent motility of fresh and cryopreserved-thawed spermatozoa was estimated. The fertility trials of cryopreserved-thawed milt were carried by following the protocol as mentioned above for optimizing the sperm egg ratio trials.

Morphological studies of fresh and cryopreserved-thawed spermatozoa

Morphological studies of fresh and cryopreserved-thawed spermatozoa were done using Philips-make Scanning Electron Microscopic (SEM) SEM Model No. L30 at 'ICAR-Central Institute of Research on Cotton Technology (CIRCOT)', Mumbai, India as suggested by Ghadially (1986). Milt samples were fixed in chilled 3% glutaraldehyde solution for 1 to 2 hours at 4^0C and a fresh change of cold glutaraldehyde was given at the end of fixation. The samples were then washed in cold 0.1M sodium cacodylate buffer over half an hour with three to four changes to ensure removal of excess glutaraldehyde. Post fixation with OsO_4 or Osmication with 1% OsO_4 in sodium cacodylate buffer was carried for 2 hours at 4^0C. Then the samples were dehydrated by passing the fixed samples through a series of graded alcohols of increasing concentration ending with absolute alcohol. The morphology of fresh spermatozoa and cryopreserved-thawed spermatozoa were studied. The morphometric values for fresh spermatozoa were recorded randomly for 100 spermatozoa which included head diameter, length and width of mid-piece and tail.

Ultrastructure studies of fresh and cryopreserved-thawed spermatozoa

Ultrastructural studies of fresh and cryopreserved-thawed spermatozoa were carried using Transmission Electron Microscopy (TEM) Jeol- make, Model number, TEM Jem-1010 at 'Jaslok Hospital and Research Center', Mumbai, India as per Ghadially (1986). Both the fresh and cryopreserved - thawed milt samples were fixed in 3% glutaraldehyde (in 1*M* Na-Cacodylate buffer) for 6-8 hours at refrigeration temperature, washed with sodium-cacodylate four times at 5 minute intervals, again fixed with 1% osmium tetroxide (in D/W) and 1*M* sodium cacodylate in the ratio of 1:1 (v/v) for 1-2 hours at refrigeration temperature. Samples were then washed with 1*M* sodium cacodylate buffer for 10 minutes followed by dehydration schedule with ethyl alcohol / acetone. Semi-thin sections from the tissue blocks, stained with toluidine blue were observed under 40 X. The selected areas were marked for utlra-thin sectioning. The ultra-thin sections were taken over copper grids and

stained with lead citrate and uranyl acetate and subsequently were loaded into TEM for observation and photomicrography. The ultrastructural details of fresh spermatozoa were recorded. A minimum of 100 spermatozoa were counted and the percentage of damaged spermatozoa was estimated.

Sperm mitochondrial activity index (SMAI) assessment by Nitro Blue Tetrazolium (NBT) assay

Sperm Mitochondrial Activity Index (SMAI) assessment using Nitro Blue Tetrazolium (NBT) assay was conducted for evaluation of physiological changes in the cryopreserved-thawed spermatozoa (Stasiak and Bauman 1996; Gopalakrishnan *et al.*, 1998). The activity of the mitochondrial enzyme-system was measured in terms of reduction of the dye NBT into formazan precipitate. Based on the intensity of the color developed, the enzyme activity were quantified. Aliquots of the cryopreserved –thawed milt as well as the fresh milt were centrifuged in refrigerated centrifuge at 2,000 rpm for 10 minutes. The spermatozoa pellet was resuspended in 0.5 ml sterile, phosphate buffered saline (PBS). About 100 µl of resuspended sperm cells were placed into each well of the 96-well flat bottom micro-titre plates and incubated at 37°C for 1 hour and supernatant was removed. About 100µl of 0.3% freshly prepared NBT in PBS was added to each well and incubated at 37° C for 1 hour and excess NBT was discarded. The cells were fixed with 100 µl of 100% methanol in each well and incubated for 5 minutes. Wells were washed with 100µl of 70% methanol, three times and excess methanol was discarded and the wells were air dried for 15-20 minutes. 120µl of 2*M* KOH and 140µl of DMSO were added into each well to dissolve the formazan blue precipitate. The resulting turquoise blue colored solution was read using ELISA reader (Lab Systems Multiskan MS) at a wavelength of 620 nm using "Genesis" software version 3.03.

Statistical analysis

Normality of the data was tested and wherever needed appropriate transformation methods were used to bring the data to normal distribution. Analysis of Variance was performed to estimate the significant differences between various treatment groups. All statistical analyses were performed using SAS Statistical Analysis Package (Version 8.2).

RESULTS AND DISCUSSION

Estimation of spermatozoa density and motility percentage

The mean sperm density in the fresh milt was $3.96 \pm 0.12 \times 10^7$ spermatozoa/ml (Table 1). In contrast to the present findings, higher sperm concentrations of $1.70 \pm 0.29 \times 10^9$ sperm cells/ ml were reported in *T. putitora* (Agarwal, 2011) and $2.58 \pm 0.16 \times 10^{10}$ sperms per ml was reported in rohu (Jahageerdar *et al.*, 2007). However, similar results with sperm densities ranging between 2.0 to 2.5×10^7, 3.0 to 3.25×10^7 and 2.0 to 2.5×10^7 cells / ml

were reported for catla, rohu and mrigal respectively (Gupta and Rath, 1996).

The average motility percentage of spermatozoa from fresh milt samples was estimated to be 95.18 ± 0.29 (Table 1). Similar percent of motility in fresh milt was reported in *T. khudree* by Basavaraja *et al*. (2002). The sperm concentration and motility is influenced by several factors viz., species, feeding conditions, husbandry practices, age, body weight, environmental factors and spawning time, dilution ratio (Tahoun *et al*., 2008; Aliniya *et al*., 2013).

(2002), Jahageerdar *et al*. (2007) had reported a lower optimum sperm: egg ratio, in *T. khudree and Labeo rohita*, respectively. On the contrary, higher optimal sperm: egg ratios of 1.8-2.4 x 10^5 and 3.38 x 10^7 spermatozoa /egg in case of common carp and brown trout, respectively were reported (Gopalakrishnan *et. al.,* 1999; Linhart *et. al*., 2000). These differences may be due to species variation, environmental factors, diet and health condition of fish and others (Gopalakrishnan *et al*., 1999).

Table1. Density of spermatozoa and motility percentage of the fresh milt of *T. putitora*

Sperm Cell Density (x10^7)	Mean Cell Density (x10^7) (Mean± SE)	% Motile Spermatozoa	Mean Percent Motile Spermatozoa (Mean ± SE)
4.05		94.69	
4.68		54.49	
3.95		96.32	
4.16		95.96	
3.6		49.37	95.18±0.29
4.44	3.96 ± 0.12	94.50	(only from nine males
3.35		96.41	with ⩾ 70% motility)
4.19		94.93	
3.21		95.16	
3.87		93.94	
4.22		56.54	
3.82		94.72	

Optimization of sperm: egg ratio

The mean hatching percentages for fresh milt at various dilutions are given in Table 2. In the present study the optimum sperm: egg ratio was estimated to be 3.96 x 10^4 /egg. Basavaraja *et al*.

Evaluation of the fertility of cryopreserved-thawed spermatozoa

Higher fertilization and lower hatching rates were observed for the cryopreserved spermatozoa (Fig. 1). The average hatching percentage with

cryopreserved-thawed milt was 45.97 ± 1.72 which was significantly lower than the average hatching percentage of 73.10 ± 0.82 for fresh milt (Table 2, Fig.1).

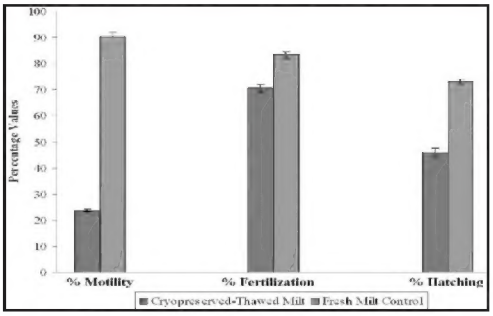

Figure 1. Percentage motility, fertilization and hatching for cryopreserved-thawed spermatozoa

The decrease in fertilization and hatching percentage by using cryopreserved milt as compared to the use of neat milt is expected. The cryopreservation-thawing leads to various types of cryoinjuries to spermatozoa and it reduces both the number of live spermatozoa available for fertilization and also their motility and capacity to fertilize because of which the fertilization rate drops (Table 4). In the present study, it was observed that the hatching rates were lower when compared to the fertilization rates. Probably some spermatozoa whose DNA was damaged may still had entered the egg through micropyle and may had fertilized the eggs and initiated the cell division, but later due to the presence of damaged DNA the subsequent embryonic development may had got arrested leading to lower hatchability rate (Jamiesion and Leung, 1991; Gwo *et al.,* 2003). Similar observations were reported for cryopreserved-thawed spermatozoa with lower hatching rate when compared to higher fertilization rate in *T. khudree* (Basavaraja *et al.,* 2002; Patil and Lakra, 2005). Contrary to the results of the present study, a lower fertilization rate of 23.8% and a lower hatching rate of 13.6% was reported in *T. putitora* (Ponniah *et al.,* 1999).

Morphological and ultrastructure studies

The morphological study of the fresh spermatozoa of *T. putitora* using SEM,

Table2. Hatching percentages at different fresh milt dilutions in *T. putitora*

Dilutions	Replications			Mean Percent Hatching (Mean ± SE)
	R1**	R2	R3	
Neat Milt	76.42	80.22	75.25	77.29±1.50[a*]
10-1	75.00	75.65	71.57	74.07±1.27[a*]
10-2	67.62	64.65	72.04	68.10±2.15[a]
10-3	39.53	45.37	38.14	41.02±2.21[b]
10-4	39.66	39.81	38.95	39.47±0.26[b]

***** Values with the same superscripts are not significantly different

** R_1, R_2, R_3 are the replications

revealed a spherical head followed by a not-distinct mid-piece and a long tail (Fig. 2). The head diameter was 1.97 ± 0.016μm, the length of mid piece and tail were 0.31 ± 0.003μm and 31.10±0.133μm, respectively (Table 3). The reviewed literature suggested that there are no earlier work on morphological study of *T. putitora* spermatozoa. Larger sperm head diameter ranging from 6.41 to 7.35, were reported in common carp (Chutia *et al.,* 1998). However, the sperm tail in common carp reported by Chutia *et al.* (1998) was found to be similar to the present study.

Figure 2. Scanning Electron Micrograph of fresh spermatozoa of *Tor putitora* (x 2,500): ha - head; mp - mid-piece; ta - tail

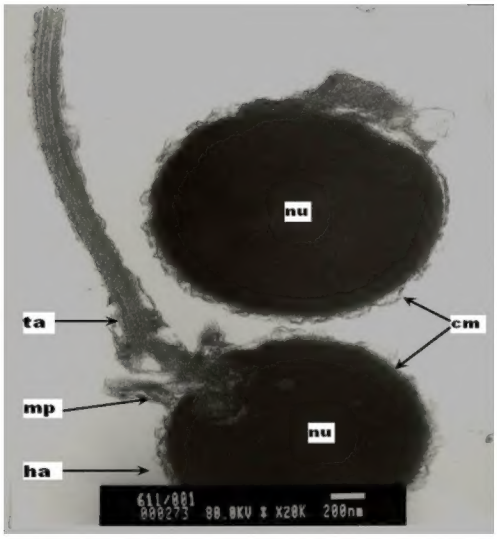

Figure 3. Transmission Electron Micrograph of fresh spermatozoa of *T. putitora* (x 20,000): ha - head; mp - mid-piece; ta - tail; nu - nucleus; cm - cytoplasmic membrane; ce- cytoplasmic extension

The ultrastructure studies of fresh spermatozoa by TEM, also revealed spherical head and not-so-distinct mid-piece (Fig. 3). It also showed posterior end of the head with a basal plate forming the origin of the tail (Fig. 4). Cross section of the tail showed a typical nine pairs of peripheral and a single pair of central doublet micro-tubules (Fig. 5). Similar ultrastructure of fresh spermatozoa were reported in *Gymnotus carapo* (Vergilio *et. al*., 2012) and *Sander lucioperca* (Kristian *et al.*, 2014).

Table 3. Morphometric characteristics of fresh spermatozoa by Scannning Electron Microscopy (SEM) in *T. putitora*

Head	Mid-piece		Tail	
Diameter (in μ m) (Mean ± SE) (n= 50)	Length (in μ m) (Mean ± SE) (n=50)	Width (in μ m) (Mean ± SE) (n= 50)	Length (in μ m) (Mean ± SE) (n=50)	Width (in μ m) (Mean ± SE) (n= 50)
1.97 ± 0.016	0.31±0.003	0.62±0.002	31.10±0.133	0.30±0.003

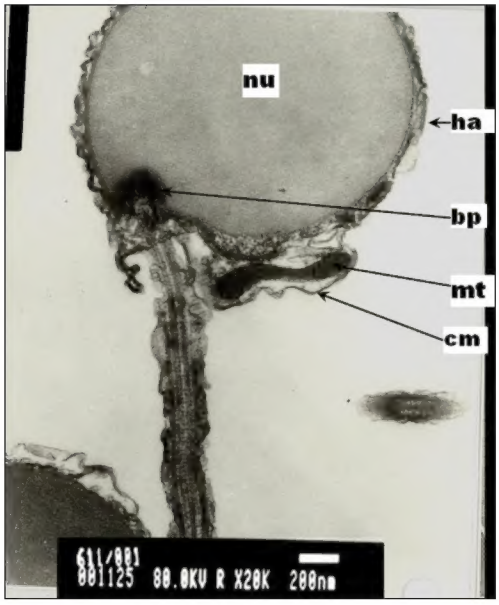

Figure 4. Transmission Electron Micrograph of fresh spermatozoa of *T. putitora* (x 20,000): ha - head; nu - nucleus; cm - cytoplasmic membrane; bp- basal plate; mt - mitochondria

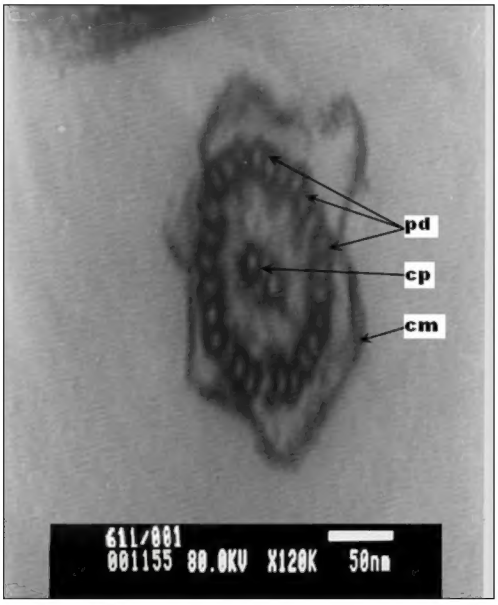

Figure 5. Transmission electron micrograph of fresh spermatozoa of *T. putitora* showing cross section of the tail (x1,20,000): pd- peripheral doublets; cp- central pair; cm - cytoplasmic membrane

SEM study of cryopreserved-thawed spermatozoa showed several morphological deformities viz., loss of tail, curving of the tail, shrunken mid-piece, appearance of verrucosities on the sperm head and severe roughening of surface of the head (Fig. 6). Different authors also had observed similar morphological deformities in cryopreserved–thawed spermatozoa of different carp species under SEM observations (Saad *et. al.,* 1988; Wei-Xin and Ren-Liang, 1991; NBFGR, 1998).

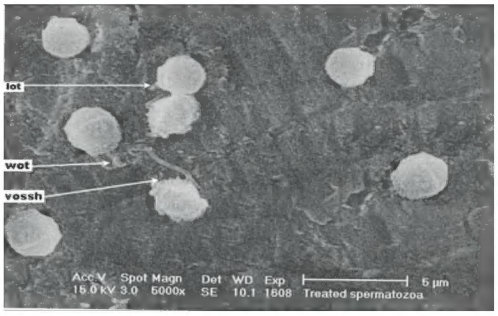

Figure 6. Scanning Electron Micrograph of cryopreserved-thawed spermatozoa of *T. putitora* showing various kinds of damage (x 5,000) : lot- loss of tail; wot- winding of tail; vossh - verrucosities on the surface of sperm head

The TEM analysis of cryopreserved-thawed milt revealed that 54.43 percent damaged spermatozoa while it was only 1.80 percent in fresh milt (Table 4). The damaged spermatozoa exhibited amoeboid, triangular or irregular shapes, loosened chromatin material and disrupted cytoplasmic membrane (Fig. 7 and 8). Cryopreservation induces cellular

damages, however, standardizing the freezing techniques helps to minimize the cryoinjuries to the sperms. High percentage damage to sperms during cryopreservation was observed in *Labeo rohita* (NBFGR, 1998). The earlier studies in various fish species also observed ultrastructural damages in fish spermatozoa following cryopreservation-thawing cycle (Saad *et. al.,* 1988; NBFGR, 1998; Drokin *et. al*., 2003).

SMAI assessment using NBT assay

In the present study, NBT assay was employed for the first time in case of fish spermatozoa for Sperm Mitochondrial Activity Index assessment. SMAI is an index used for measuring the functional integrity of mitochondrial enzyme complex. NBT assay is routinely used for SMAI assessment in human spermatozoa for evaluation of fertility

Table 4. Percent average of damaged spermatozoa in fresh and cryopreserved-thawed milt using Transmission Electron Microscopy (TEM) in *T. putitora*

Milt Sample	Percentage of Damaged Spermatozoa (Mean ± SE)
Fresh milt	1.80 ± 0.19
Cryopreserved-thawed milt	54.43 ± 2.05

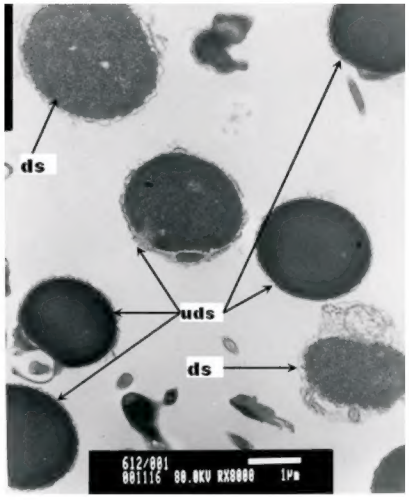

Figure 7. Transmission Electron Micrograph showing the damaged and undamaged spermatozoa during the thawing (x 8,000): uds - undamaged spermatozoa; ds - damaged spermatozoa

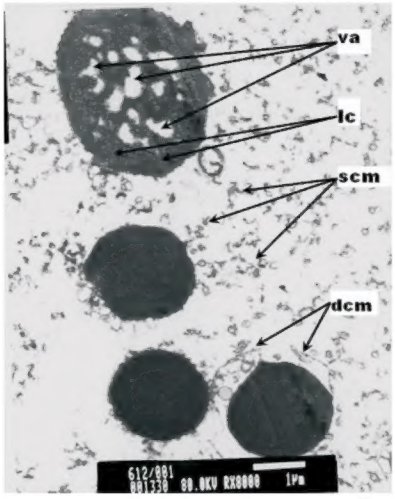

Figure8. Transmission electron micrograph showing the damage caused to the spermatozoa of *T. putitora* due to thawing (x 8,000): va- vacuolization; lc- loosening of chromatin; dcm- disrupted cytoplasmic membrane; scm- spilled chromatin material

Table 5. Absorbance values(OD) of spermatozoa in SMAI assessment by NBT assay in *T. putitora*

Milt Sample	Cryopreserved-thawed milt	Fresh milt (Control)
R1	0.137	0.322
R2	0.152	0.357
R3	0.148	0.321
Mean± SE	0.15±0.005[a]	0.33±0.012[b]

* Values with different superscripts are significantly different

status (Bergstrom and Jarkman, 2013). In the present study the absorbance values of cryopreserved-thawed spermatozoa were significantly lower than those of fresh spermatozoa indicating a definite loss in the functional integrity of the enzymes from the mitochondrial enzyme complex (Table 5). During cryopreservation-thawing, damages may have been caused to the structural mitochondrial enzyme complex of spermatozoa which may have resulted in loss of functional activity of these enzymes resulting in low enzyme activity. Mitochondrial enzyme complex of the spermatozoa plays an important role in ATP generation which gives energy required for the motility of spermatozoa to fertilize the eggs. Hence it is necessary to maintain the structural and functional integrity of the enzymes of the mitochondrial enzyme complex of the spermatozoa. The present study indicates that SMAI may be employed as an additional biochemical test to assess the accuracy of cryopreservation and also as an indicator of fertility status of fish milt.

CONCLUSION

The spermatozoa of *T. putitora* are having a spherical head followed by a not-distinct mid-piece and a long tail. The optimum sperm: egg ratio was estimated to be 3.96 x 10^4 /egg. The present study further confirmed that cryopreservation-thawing results in cryo-injuries to the spermatozoa and the ultrastructural damages include disruption of nucleus, tail as well as reduced or loss of activity of the mitochondrial enzyme complex. The present study also found that SMAI may be employed as an additional biochemical test to assess the accuracy of cryopreservation and also as an indicator of fertility status of fish milt.

ACKNOWLEDGEMENTS

The financial assistance received from Indian Council of Agricultural Research, New Delhi in the form of Senior Research Fellowship to the first author for the present work is gratefully acknowledged. The authors thank the Director, CIFE, Mumbai and the Environmental Officer, Tata Power Company, Lonavala for providing the facilities to carry this research.

REFERENCES

Agarwal, N. K., 2011. Cryopreservation of fish semen. In : M. Thapliyal, A.

Thapliyal and J. P. Bhatt (Eds.) Himalayan Aquatic Biodiversity Conservation & New Tools in Biotechnology. Transmedia Publication, Srinagar (Garhwal), Uttarakhand, pp. 104-127.

Aliniya, M., H. Khara, S. B. Noveiri and H. Dadras, 2013. Influence of age of common carp (*Cyprinus carpio*) broodstock on reproductive traits and fertilization. *Turk. J. Fish. Aquat. Sci.*, 13: 19-25.

Ax, R. L., M. Dally, B. A. Didion, R. W. Lenz, C. C. Love, D. D. Varner, B. Hafez and M. E. Bellin, 2000. Semen evaluation. *In* : E. S. E. Hafez and B. Hafez (Eds.) Reproduction in farm animals. Lippincott Williams and Wilkins, New York, pp. 365-375.

Basavaraja, N., S. N. Hegde, N. Akash and K. S. Udupa, 2002. The fertility of cryopreserved deccan mahseer, *Tor khudree* (Sykes) spermatozoa. *Asian Fish. Sci.*, 15: 193-202.

Basavaraja, N. and P. Keshavanath, 2000. Conservation and management of fish genetic resources in Karnataka. *In* : A. G. Ponniah and A. Gopalakrishnan (Eds.) Endemic fish diversity of Western Ghats, NBFGR, Lucknow, pp.152-154.

Biggers, J. D., W. K. Whitten and D. G. Whittingham, 1971. The culture of mouse embryos in vitro, In: Methods in Mammalian Embryology. (Table 6-5). (J. C. Daniel% ed.). Freeman, San Francisco. pp. 86-116.

Bergstrom, B. and S. Jarkman, 2013. Correlation between sperm oxidative stress and sperm DNA damage in subfertile men. Masters Thesis, Lund University, Sweden, 34 pp.

Billard, R., J. Cosson and O. Linhart, 2000. Changes in the flagellum morphology of intact and frozen/thawed Siberian sturgeon *Acipenser baerii* (Brandt) sperm during motility. *Aquaculture Research*, 31: 283–287.

Butts, I. A. E., R. M. Rideout, K. Burt, S. Samuelson, L. Lush, M. K. Litvak, E. A. Trippel and D. Hamoutene, 2010. Quantitative semen parameters of Atlantic cod (*Gadus morhua*) and their physiological relationships with sperm activity and morphology. *J. Appl. Ichthyol.*, 26: 756–762.

Chao, N. H. and I. C. Liao, 2001. Cryopreservation of finfish and shellfish gametes. *Aquaculture,* 197: 161-189.

Chutia, I. P., G. Krishna and A. Chaudhary, 1998. Biochemical and biometrical analysis of carp milt. *In* : A. G. Ponniah, P. Das and S. R. Verma (Eds.) Fish genetics and biodiversity conservation, Nature Conservators, Muzaffarnagar, pp. 203-213.

Cosson, J., 2012. ATP, the sperm movement energizer. *In* : E. Kuestler and G. Traugott (Eds.) Adenosine Triphosphate: Chemical properties, Biosynthesis and Functions in cells, Nova Publisher Inc., New York, pp. 1-46.

Drokin, S. I., H. Stein, T. P. Govorukha, 2003. Ultrastructure of carp, *Cyprinus carpio* spermatozoa after cooling, dilution and freeze-thawing. *CryoLett.*, 24: 49-56.

Figueroa, E., I. Valdebenito, A. B. Zepeda, C. A. Figueroa, K. Dumorne, R. L. Castillo and J. G. Farias, 2015. Effects of cryopreservation on mitochondria of fish spermatozoa. *Rev. Aquaculture*, 08/2015;DOI:10.1111/raq.1210.

Ghadially, F. N., 1986. Processing of tissue for routine electron microscopy. *In* : F. N. Ghadially (Ed.) Course on diagnostic Electron Microscopy, Jaslok Hospital and Research Center, Mumbai, pp. 1-7.

Gopalakrishnan, K., I. N. Hinduja, R. H. Mehta and T. C. A. Kumar, 1998. Human semen analysis. *In* : K. Gopalakrishnan, I. N. Hinduja, R. H. Mehta and T. C. A. Kumar (Eds.) Laboratory manual for human semen analysis. Institute for Research in Reproduction and WHO Collaborating Center, Mumbai, pp. 1-34.

Gopalakrishnan, A., K. L. Thakur, A. G. Ponniah, K. Kumar and R. Dayal, 1999. Cryopreservation of Brown Trout (*Salmo trutta fario*) sperm: the influence of extender composition and fertilization procedure. *Fish. Technol.,* 36: 104-109.

Gupta, S. D and S. C. Rath, 1996. Cryogenic preservation of carp milt and its utilization in seed production. *In* : Proceedings of the third Indian Fisheries Forum, Asian Fisheries Society Indian Branch, Mangalore, pp. 77-79.

Gwo, J. C., C. Y. Wu, W. S. P. Chang and H. Y. Cheng, 2003. Evaluation of damage in Pacific oyster (*Crassostrea gigas*) spermatozoa before and after cryopreservation using comet assay. *CryoLett.,* 24: 171-180.

Hatef, A., S. M. H. Alavi, Z. Linhartova, M. Rodina, T. Policar and O. Linhart, 2010. Effects of Bisphenol A on sperm motility characteristics in *Perca fluviatilis* (Perciade; Teleostei). *J. Appl. Ichthyol.,* 26: 696-701.

Jahageerdar S. Gopalkrishna and J.K. Prasad, 2007. Effective spermatozoa to egg ratio for artificial insemination in Labeo rohita (Ham.). *Indian J. Fish.,* 54*(1): 107-115.*

Jamieson, B. G. M. and L. K. P. Leung, 1991. Introduction to fish spermatozoa and the micropyle. *In* : B. G. M. Jamieson (Ed.) Fish evolution and systematics: Evidence from spermatozoa. Cambridge University Press, Cambridge, pp. 56-72.

Kristian, J., A. Hatef, S. M. H. Alavi and T. Policar, 2014. Sperm morphology, ultrastructure, and motility in pikeperch *Sander lucioperca* (Percidae, Teleostei) associated with various activation media. *Czech J. Anim. Sci.,* 59: 1–10.

Linhart, O., M. Rodina and J. Cosson, 2000. Cryopreservation of sperm in common carp, *Cyprinus carpio*: sperm motility and hatching success of embryos. *Cryobiology,* 41: 241-250.

Mahanta, P. C., D. Kapoor, A. K. Pandey, S. M. Srivastava, R. Dayal, R. S. Patiyal, K. D. Joshi, A. K. Singh and S. K. Paul, 1988. Conservation and rehabilitation of mahseer in India. *In* : A. G. Ponniah, P. Das and S. R. Verma (Eds.) Fish genetics and biodiversity conservation. Nature Conservators, Muzaffarnagar, pp. 93-105.

NBFGR, 1998. Electron Microscopy studies of cryopreserved spermatozoa of *Labeo rohita*. *In* : NBFGR (Ed.) Annual Reports. National Bureau of Fish Genetic Resources, Lucknow, pp. 28-31.

NBFGR, 2000. Threatened fishes of India. *In* : NBFGR (Ed.) Annual Reports. National Bureau of Fish Genetic Resources, Lucknow, pp. 16-17.

Ogale, S. N., 1994. Endangered deccan mahseer, *Tor khudree* (Sykes)- A case study. *In* : P. V. Dehadrai, P. Das and S. R. Verma (Eds.) Threatened fishes of India. Nature Conservators, Muzaffarnagar, pp. 213-218.

Patil, R. and W. S. Lakra, 2005. Effect of different cryoprotectants, equilibration periods and freezing rates during cryopreservation of spermatozoa of mahseer, *Tor khudree* (Sykes) and *T. putitora* (Hamilton). *Aquaculture Res.*, 36:1465–1472.

Ponniah, A. G., W. S. Lakra and S. N. Ogale, 1999. Effects of cryoprotectant and fertilization protocol on viability of mahseer *Tor khudree* cryopreserved spermatozoa. *J Aquacult. Trop.,* 14: 153-158.

Rao, K. G., 1989. Cryopreservation of carp sperm. *In* : P. Das and A. G. Jhingran (Eds.) Fish genetics in India. Today's and Tomorrow's Printers and Publishers, New Delhi, pp. 193-198.

Ravinder, K., K. Nasaruddin, K. C. Majumdar and S. Shivaji, 1997. Computerized analysis of motility, motility patterns and motility

parameters of spermatozoa of carp following short-term storage of semen. *J. Fish Biol.,* 50: 1309-1328.

Saad, A., R. Billard, M. C. Theron and M. G. Hollebecq, 1988. Short- term preservation of carp (*Cyprinus carpio*) semen. *Aquaculture,* 71: 133-150.

Stasiak, S. A. and P. C. Bauman, 1996. Neutrophil activity as a potential bioindicator for contaminant analysis. *Fish and Shellfish Immunol.,* 6: 537-539.

Suquet, M., R. Billard, J. Cosson, Y. Normant and C. Fauvel, 1995. Artificial insemination in turbot (*Scophthalmus maximus*): determination of the optimal sperm to egg ratio and time of gamete contact. *Aquaculture*, 133: 83-90.

Tahoun, A. M., M. A. R. Ibrahim, Y. F. Hammoudi, M. S. Eid, M. M. A. Z. El-Din and F. I. Magouz, 2008. Effect of age and stocking density on spawning performance of nile tilapia, *Oreochromis niloticus* broodstock reared in hapas. *In* : Proceedings of 8th International Symposium on Tilapia in Aquaculture, The Central Laboratory for Aquaculture Research, Cairo, pp.329–343.

Vergilio, C. D. S., R. V. Moreira, C. E. V. D. Carvalho and E. S. J. T. Melo, 2012. Characterization of mature testis and sperm morphology of *Gymnotus carapo* (Gymnotidae, Teleostei) from the southeast of Brazil. *Acta Zoologica*, 0: 1–7.

Wei-Xin, Z. and J. Ren-Liang, 1991. Freezing damage to sperm and embryos of carp. *Asian Fish. Sci.,* 4: 630-635.